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Expression of *bkt* and *bch* genes from *Haematococcus pluvialis* in transgenic *Chlamydomonas*

ZHENG KaiJing†, WANG ChaoGang†, XIAO Ming, CHEN Jun, LI JianCheng & HU ZhangLi*

Shenzhen Key Laboratory of Marine Bioresource & Eco-environmental Science, Shenzhen Engineering Laboratory for Marine Algal Biotechnology, College of Life Science, Shenzhen University, Shenzhen 518060, China

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β-carotene ketolase and β-carotene hydroxylase encoded by *bkt* and *bch*, respectively, are key enzymes required for astaxanthin biosynthesis in *Haematococcu pluvialis* 34-1n. Two expression vectors containing cDNA sequences of *bkt* and *bch* were constructed and co-transformed into cell-wall-deficient *Chlamydomonas reinhardtii* CC-849. Transgenic algae were screened on TAP agar plates containing 10 μg mL^{-1} Zeomycin. PCR-Southern analysis showed that *bkt* and *bch* were integrated into the genomes of *C. reinhardtii.* Transcripts of *bkt* and *bch* were further confirmed by RT-PCR-Southern analysis. Compared with the wild type, transgenic algae produced 29.04% and 30.27% more carotenoids and xanthophylls, respectively. Moreover, the transgenic algae could accumulate 34% more astaxanthin than wild type. These results indicate that foreign *bkt* and *bch* genes were successfully translated into β-carotene ketolase and β-carotene hydroxylase, which were responsible for catalyzing the biosynthesis of astaxanthin in transgenic algae.

***Chlamydomonas*, β-carotene ketolase, β-carotene hydroxylase, carotenoids, xanthophylls, astaxanthin**

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Astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione), a member of the carotenoid family, has been shown to be an efficient antioxidant, enhancer of immune responses, anti-aging and anti-cancer agent [1]. Studies on human safety indicated that astaxanthin was not toxic to adults [2]. Astaxanthin has also been used as a component in cosmetics and nutritional products. It is also an indispensible feed additive for salmon and trout aquaculture [3]. Only a few species, including green algae, fungi and bacteria, can synthesize astaxanthin *de novo*. The needs for astaxanthin in most animals are supplied from food intake. Supply of natural astaxanthin cannot meet the demand in the international market. Though crustaceans and the green algae *H. pluvialis* and *P. rhodozyma* have been developed to produce astaxanthin, the output of astaxanthin is still not enough. Although the synthetic astaxanthin is much cheaper, the structural properties, applications and safety are very different from its natural counterpart [4]. Because of these limitations, it is of great interest to improve the biosynthesis of natural astaxanthin through genetic manipulation.

Over the past 15 years, genes coding for carotenoid biosynthetic enzymes have been cloned. β-carotene ketolase and β-carotene hydroxylase are two key enzymes responsible for the conversion of β-carotene to astaxanthin in marine bacteria and *H. puvialis*. The functions of these two enzymes, along with several carotenogic genes from *Erwinia uredovora* and *Rhodobacter* species, were demonstrated by complementary *in vivo* and *in vitro* assay systems. Different

†Contributed equally to this work
*Corresponding author (email: huzl@szu.edu.cn)

carotenoid compounds were produced when a combination of carotenogenic genes were expressed simultaneously through genetic manipulation [5,6]. β-carotene ketolase and β-carotene hydroxylase encoded by *bkt* and *crtZ* respectively were detected in *E. uredovora.* Simultaneous expression of *bkt and crtZ* can lead to the accumulation of ketocarotenoids, including astaxanthin in *Escherich coli* [5]. A similar result was reported using the *bch* from *H. puvialis* [7]. Introduction of *bkt* into cyanobacterium *Synechococcus* PCC7942 and tobacco could also result in the production of astaxanthin and other ketocarotenoids [8,9]. These studies demonstrated that the production of natural astaxanthin in other species was feasible using transgenic technology.

C. reinhardtii, another type of unicellular green alga, shares significant similarities with *H. pluvialis*. *C. reinhardtii* can be easily cultured in inexpensive media and grow rapidly. Moreover, its genetic background has been well-documented and it has three autonomous genetic systems: nuclear, chloroplast and mitochondria. Transformation of *C. reinhardtii* chloroplasts with *bch* could change photosynthetic pigments in transformants [10]. However, astaxanthin synthesis could not be realized when only *bch* is expressed in *C. reinhardtii.* In addition, chloroplast expression of *bch* also risks the loss of transgene and homogenization. Therefore, astaxanthin can not be produced abundantly by expressing *bch* in chloroplast, unless *bkt and bch* were expressed simultaneously in *C. reinhardtii*. This point was also supported by Huang et al. [11].

In the present study, both *bkt* and *bch* genes isolated from *H. pluvialis* 34-1n were co-transformed into the nuclear genome of *C. reinhardtii* CC-849. To our knowledge, this is the first report indicating the successful co-expression of *bkt* and *bch* in the nuclear genome of *C. reinhardtii*, which provided a new way for the production of natural astaxanthin by using transgenic algae.

1 Materials and methods

1.1 Strains and growth conditions

H. pluvialis 34-1n obtained from the Culture Collection of the University of Göttingen (Germany) was cultured in Bold Basal Medium media. Cells were kept at 22°C under continuous light (27 μmol m^{-2} s^{-1}) until their growth reached the logarithmic phase. To induce the expression of genes for astaxanthin biosynthesis, we added CH_3COONa and $FeSO_4$ to the media at a final concentration of 45 mmol L^{-1} and 450 μmol L^{-1} respectively, and light conditions were adjusted to 1000 μmol m^{-2} s^{-1} for 2 h. The cell-wall-deficient strain of *C. reinhardtii* CC-849 was provided by the *Chlamydomonas* Genetic Center of Duke University (USA) and was kept in Tris-acetate-phosphate (TAP) media [12].

1.2 Plasmids, bacteria and reagents

pH105, containing an *hsp70A-RBCS*$_2$ promoter and 231 bp of the *RBCS*$_2$ 3′ non-coding region, was maintained in our laboratory. The pSP124 plasmid, including *ble* as a selection marker, was kindly provided by Dr. Saul Purton (University College London, Britain).

The restriction endonuclease, TaKaRa LA Taq™, T4 DNA Ligase, pMD 18-T Simple Vector, RNA and PCR Fragment Recovery Kit Ver.2.0 were obtained from TaKaRa (Japan). The QIAprep® Spin Miniprep Kit (50) was obtained from Qiagen (USA).

1.3 Experimental methods

1.3.1 Nucleic acids preparation

Total RNA was extracted using TRIzol Reagent (Invitrogen, USA) according to the manufacturers' instructions. Genomic DNA was extracted using the method described by Zhang [13] and Wang [14].

1.3.2 Cloning cDNA fragments of bkt and bch

Two pairs of primers were designed according to cDNA sequences of β-carotene ketolase (GI: 1136638) and β-carotene hydroxylase (GI：5852869) in GenBank. The sequences of primers P1 and P2, used for *bkt* amplification, were 5′-TATCACATGCCATCCGAGTCG-3′ and 5′-TCATGCCAAGGCAGGCACC-3′. The sequences of primers P3 and P4, used in the *bch* amplification reaction, were 5′- CTAGCTAGCTCATTTGCTGCTACCACGATGCTGTC-3′ and 5′-ACGCGTCGACTTCCGCACCCTACCGCTTGGAC-3′, respectively. Total RNA of *H. pluvialis* 34-1n was extracted, and *bkt* and *bch* were amplified using cDNA as a template. PCR was done with a preheating step (3 min at 95°C), four cycles of initial amplification (97°C for 40 s, 53°C for 1 min, and 72°C for 90 s) and 29 cycles of successive amplification (95°C for 45 s, 56°C for 1 min, and 72°C for 90 s), and a final extension (10 min at 72°C). These PCR products were purified and then subcloned into pMD 18-T Simple Vector for sequencing.

1.3.3 Nuclear transformation

The transformation of *C. reinhardtii* was performed as described by Kindle [15] and Wang [14]. Algal cells were grown to mid-log phase in TAP medium and collected by centrifugation at 5000×*g* for 8 min at room temperature. The cells were then suspended in 1.5 mL tubes containing 300 μL fresh TAP medium and 1 g of 0.3 mm diameter alloy beads. One μg of linearised DNA was added and the mixture was vortexed for 25 s at top speed. The transformation mixture was transferred to a 50-mL tube containing 15 mL of TAP medium and incubated for 18–30 h. The cells were pelleted and resuspended in 0.5 mL TAP medium, kept in TAP plates containing 10 μg mL^{-1} Zeomycin at

22°C. Transformants appeared after 2–3 weeks of incubation. In order to obtain transgenic algae containing both *bkt* and *bch* genes, we added 1 μg pH105bkt124 and pH105-bch124 plasmid in the co-transformation, respectively.

1.3.4 PCR analysis of transgenic algae

Genomic DNA isolated from transgenic algal cells was used as template for PCR amplification. First, *bkt* primers (P1 and P2) were used for screening transgenic algae that contained *bkt*. After that, transgenic algae containing *bkt* were selected by PCR with *bch* primers (P3 and P4). Transgenic algae that can amplify both *bkt* and *bch* were selected as co-transformants and used in the subsequent analysis. PCR programs were the same as those described in 1.3.2.

1.3.5 Transformants confirmation by PCR and Southern hybridization

After 5 d of growth, the transgenic *C. reinhardtii* CC-849 was harvested by centrifugation at 4°C. Genomic DNA was then isolated and PCR was performed using the *bkt* and *bch* primers.

For Southern blot analysis, the PCR products were separated by electrophoresis on 1% agarose gel and transferred to a positively charged nylon membrane, and then hybridized in the presence of *bkt* and *bch* molecular probes labeled with DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, USA).

1.3.6 Verification of transgene expression

Total RNA was extracted using TRIzol Reagent. RT-PCR was carried out by using PCR Kit (AMV) Ver. 3.0 (TaKaRa) according to the manufacturer's instructions. PCR amplification of *bkt* and *bch* was done using the cDNA as templates. The PCR products were hybridized with *bkt* and *bch* DIG probes respectively.

1.3.7 Analysis of carotenoids

Algal cells were grown to mid-log phase (1×10^6 cells mL^{-1}), then exposed to high light intensity (1000 μmol m^{-2} s^{-1}) for 2 h. Cultures (50 mL) were harvested by centrifugation, and the pellet was resuspended in 30 mL methanol-KOH solution (5% KOH in 30% methanol) to destroy the chlorophyll. The supernatant was discarded, and the remaining pellet was extracted with 5 mL chloroform: ethanol (v/v=1:1), then centrifuged. The absorbance of the supernatant was determined at 400–740 nm (Molecular Devices SpectraMax M5 spectrophotometer, Molecular Devices, USA), and the amount of the carotenoids (A476), xantholphylls (A448) and astaxanthin (A490) were calculated according to Davies [16] and Huang [17].

1.3.8 Statistical analysis

All experiments were determined in biological triplicate to ensure the reproducibility. Experimental results are shown as mean ± SD.

2 Results

2.1 Isolation of *bkt* and *bch* and sequence analyses

PCR products of *bkt* and *bch* from *H. pluvialis* were inserted into a T-vector, which were further analyzed by sequencing. The *bkt* fragment was 873 bp with an opening frame of 867 bp encoding 288 amino acids. The *bch* fragment was 906 bp, containing an opening frame of 879 bp encoding a polypeptide of 292 amino acids. These sequences were 98.3% and 98.6% identical to their respective GenBank records for *bkt* and *bch* which were from *H. pluvialis* 34-1n and *H. pluvialis Flotow* NIES-144. The sequences of nucleotide and amino acid of *bkt* and *bch* were deposited to GenBank (accession numbers: DQ257290 and DQ257289).

2.2 Construction of *Chlamydomonas* expression vectors

Plasmid pH105 containing an hsp70A-$RBCS_2$ promoter and 231 bp of the $RBCS_2$ terminator, and pSP124 carrying a *ble* gene which confers zeomycin resistance of transgenic algae were used to construct *Chlamydomonas* expression vector (Figure 1). The expression vector was further confirmed by PCR analysis and restrictive digestion.

2.3 Verification of transgenic algae

Genomic DNA, prepared from transgenic algae and wild type *Chlamydomonas*, was used as template for PCR amplification. PCR products of 873 and 906 bp were observed by using the primers P1/P2 and P3/P4 respectively (Figure 2A and C). The PCR products were subjected to Southern blot analysis using the *Nhe* I-*Sal* I fragments labeled with DIG from pH105bkt and pH105bch as probes. Signals were detected at 873 and 906 bp in the DNA preparation from transgenic algae, but no signal was detected in the DNA prepared from wild type *Chlamymdomonas* (Figure 2B and D). These results indicated that *bkt* and *bch* had been integrated into the genome of *C. reinhardtii.* Transgenic algae containing *bkt* and *bch* genes were selected for the subsequent analysis.

2.4 The expression of *bkt* and *bch* genes

In order to determine the expression of *bkt* and *bch* at transcriptional level, we extracted total RNAs from transgenic C*halmydomonas* and reverse-transcribed them. Both 873 and 906 bp fragments were obtained after the amplification of *bkt* and *bch* using cDNA from transgenic algae as a template (Figure 3A and C). The RT-PCR products were further hybridized with *bkt* and *bch* probes and signals were detected (Figure 3B and D). These results showed that *bkt* and *bch* from transgenic algae could be detected in transcriptional level.

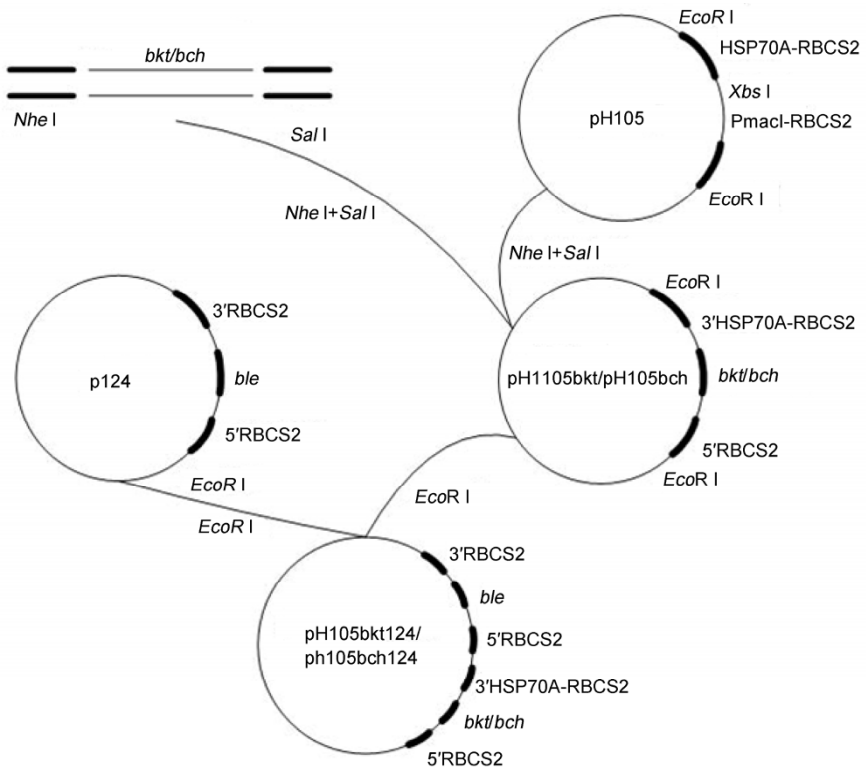

Figure 1 Diagrammatic illustration of the construction processes of the expression vector.

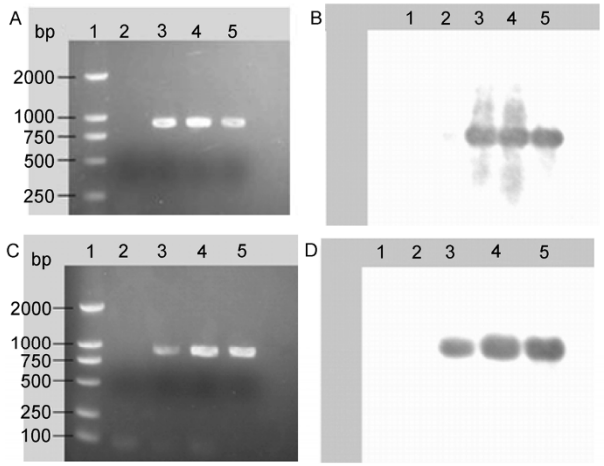

Figure 2 PCR and PCR-Southern analysis of *bkt* (A, B) and *bch* (C, D) in transgenic algae. Lane 1, marker; lane 2, wild type *Chlamydomonas*; lane 3 and 4, transgenic algae; lane 5, positive control.

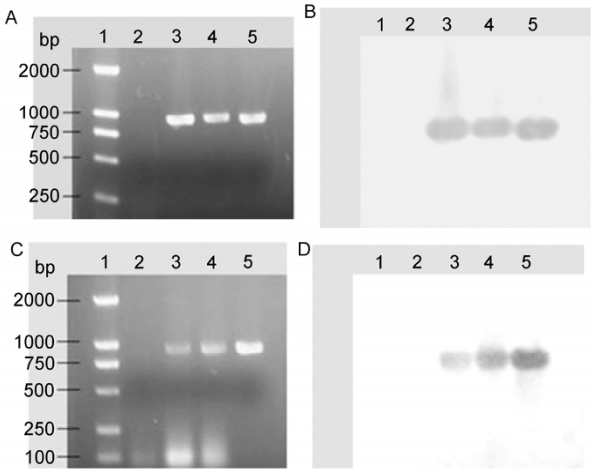

Figure 3 The expression of *bkt* (A, B) and *bch* (C, D) in transgenic algae. Lane 1, marker; lane 2, wild type *Chlamydomonas*; lane 3 and 4, transgenic algae; lane 5, positive control.

2.5 The production of carotenoids in wild type and transgenic *C. reinhardtii*

Extracts of transgenic algae were assessed for contents of carotenoids and xantholphylls. When compared with the wild type, the contents of carotenoids and xanthophylls increased by 29% and 30%, respectively (Figure 4). Although the amount of astaxanthin was low, it was also detectable. In transgenic algae, 34% more astaxanthin was found than in the wild type (Figure 5). These results demonstrated that the foreign *bkt* and *bch* genes were successfully expressed in *C. reinhardtii*. The algal cells showed additional activities of β-carotene ketolase and β-carotene hydroxylase that catalyzed the biosynthesis of astaxanthin in transgenic algae.

3 Discussion

In this study, the cloned β-carotene ketolase and β-carotene

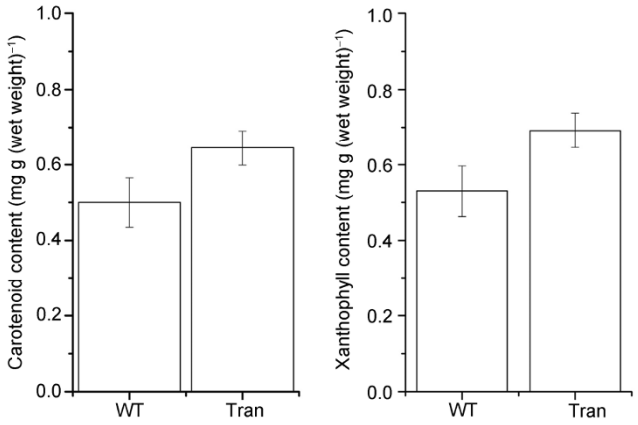

Figure 4 The amount of carotenoids and xantholphylls in wild type and transgenic strains. WT, wild type; Tran, transgenic *Chlamydomonas*.

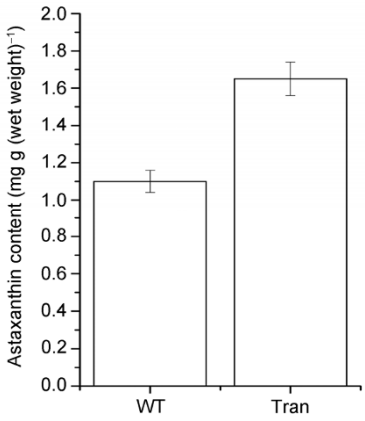

Figure 5 The accumulation of astaxanthin in wild type and transgenic *C. reinhardtii*.

hydroxylase have limited sequence similarity to bacterial sequence, which is consistent with previous literature. β-carotene ketolase from *H.piuvialis* NIES-144 shared more homology with the marine bacterial β-carotene oxygenase (36%–37%) [5], while β-carotene hydroxylase of *Arabidopsis thaliana* shared 31%–37% identity with that of bacteria [18].

β-carotene ketolase and β-carotene hydroxylase belong to the family of iron containing enzymes, and share histidine motifs for iron coordination [19]. Three conserved Fe^{2+} motifs were found in β-carotene ketolase of *H. piuvialis* and marine bacteria [20]. In the case of β-carotene hydroxylase, four highly conserved histidine residues were found in prokaryote, green algae and plants [7]. These conserved histidine motifs were also found in the two enzymes from *H. piuvialis* 34-1n.

The cell-wall-deficient *C. reinhardtii* CC-849 was selected in our study as a host strain to express *bkt* and *bch* due to the easiness to extract an accumulated product. In comparison to Tan et al. [10], who had attempted to synthesize astaxanthin in *C. reihhardtii* 137cc, the presence of the cell wall in *C. reihhardtii* 137cc obviously increased the cost of the astaxanthin extraction. Although many foreign genes were transformed successfully into *C. reinhardtii* [14,21], the expression of exogenous genes in *C. reinhardtii* is still confronted by its complex regulation system, bias of special codons and methylation of exogenous genes [22]. Besides, "Gene silencing" and "gene separating" phenomena were observed in transgenic *C. reinhardtii* [23]. Our results demonstrated that *bkt* and *bch* cloned from *H. pluvialis* 34-1n were successfully integrated into the genome of *C. reinhardtii* and could be detected by RT-PCR at transcription level, indicating that *bkt* and *bch* were transcribed. The expression of *bch* in chloroplast was also challenged by potential homogenization and gene loss. However, transgenes could be stably integrated and expressed in the nuclear genome so that it is more beneficial to synthesize and accumulate astaxanthin in cytoplasm. We have obtained inducible promoters (*Hsp70A* and *bkt1*), which were suitable for exogenous gene expression [21,24]. These promoters provided more choices for future expression optimization.

An interesting phenomenon was observed during the present study, that is, instead of suspending in the culture, one of the transgenic strains preferred staying at the bottom of an Erlenmeyer flask. Possible reasons are: firstly, the introduction of exogenous genes might influence the growth of transgenic *C. reinhardtii.* Secondly, foreign gene inserted into the middle of the genes which were responsible for movement in *C. reinhardtii.* Further experiments would be required to address this question.

Astaxanthin is valuable in aquaculture, food, medicine and cosmetics industry. Since synthetic astaxanthin has some defects and the production of natural astaxanthin is limited, the expression of *bkt* and *bch* in C*hlamydomonas* can serve as a platform for producing astaxanthin and other important carotenoids. A new report confirmed that the production of astaxanthin in tomato could be achieved by co-expression of *bkt* and *bch* genes [11]. The production of genetically engineered astaxanthin is commercially valuable. It is also beneficial to use *C. reinhardtii* due to its rapid growth and cell-wall-dificient strain.

Although transgenic strains of *C. reinhardtii* have been obtained, it is still very necessary to increase the expression level of exogenous genes. More work should be conducted to optimize the expression of foreign genes in order to improve astaxanthin content in transgenic *C. reinhardtii*.

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